of vitamin K from other lipids. In this way, TLC on silica plates developed with light petroleum ether–diethyl ether (85 : 15, by volume) is included in the sample preparation for the determination of vitamin K in lipid-rich animal tissues. Although no recent publications have been found, TLC on silica plates is especially suited for the separation of geometric isomers (*cis-trans* isomers).

Silica plates have been impregnated with 5–20% silver nitrate. Under these conditions lipids containing unconjugated double bonds in their side chain form complexes with the silver ions and show a higher retention than the saturated counterparts. Consequently, separation between saturated ($K_{1(20)}$), partly saturated [MK-*n* (H_n)] and fully unsaturated homologues (MK-*n*) becomes possible. On the other hand, in argentation chromatography the resolution between *cis* and *trans* isomers is completely lost. Silver ions are not destructive for vitamin K, so samples can be eluted from the silica afterwards. However, for high molecular weight menaquinones, irreversible adsorption to argentation TLC plates has been reported.

Unlike in argentation TLC, where retention is correlated to the degree of unsaturation, in reversed-phase TLC the retention is based on the length of the side chain. Both techniques are thus perfectly complementary for the separation of menaquinones.

In addition to silica plates and argentation TLC, reversed-phase TLC has been applied to vitamin K-related compounds. Typical eluents consist of water and an organic solvent such as methanol, acetonitrile or tetrahydrofuran. However, because of wettability problems with aqueous solvents, often nonaqueous reversed-phase conditions are used with dichloromethane and methanol (70 : 30, by vol.) as eluting solvent.

Detection

As with the other fat-soluble vitamins, fluorescence quenching can be applied to localize the position of vitamin K-related compounds on a TLC plate. More sensitive but often destructive for the compounds of interest include spray reagents such as 70% perchloric acid (5–10 min at 105°C), a 0.05% solution of rhodamine B in ethanol, a 0.2% anilinonaphthalene sulfonic acid solution in methanol and a 10% solution of phosphomolybdic acid in ethanol.

Again densitometry (based on reflectance, transmission) has completely replaced visual inspection as well as the offline quantification after elution of the bands. Densitometry allows internal standardization and results in a higher degree of sensitivity and speed of analysis.

General Conclusions

From the above overview it should be clear that TLC is no longer the method of choice for the analysis of fat-soluble vitamins. The major reason for this lies in the great progress made in HPLC. Newer trends such as HPTLC and densitometric scanning may give TLC a new momentum but never to the extent that it will again supersede HPLC as a routine technique for the determination of fat-soluble vitamins in foods or biological materials. Undoubtedly, however, modern instrumental TLC can offer automation, improved repeatability and more accurate quantification compared to classical TLC.

See also: **II/Chromatography: Thin-Layer (Planar):** Spray Reagents. **III/Vitamins:** Liquid Chromatography.

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Further Reading

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Liquid Chromatography

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Introduction

Vitamins are a group of organic compounds essential to life in very low concentrations. They are either

insufficiently produced by the body or not at all. Inadequate vitamin intake causes deficiency disorders in both humans and animals. The various vitamins are not related to each other chemically and have quite different properties. Two main groups, the fat-soluble and the water-soluble vitamins, may be distinguished.

Increased interest in vitamin research, together with the requirements of food and pharmaceutical quality control, have led to a proliferation of methods for vitamin assay, especially by liquid chromatography (LC). Bioassay methods are no longer used, but microbiological methods, physicochemical methods and chromatographic procedures (thin-layer chromatography, gas chromatography and liquid chromatography) are commonly employed. Classical open-column liquid chromatography is occasionally used, but modern high performance liquid chromatography (HPLC) is by far the technique of choice for vitamin analysis and is the subject of this article.

Vitamin analysis is performed to establish the vitamin status of humans or animals, to determine the potency of foods and feeds, and to monitor the storage stability of vitamin-containing pharmaceutical preparations. Information on the physicochemical and biochemical aspects of vitamins and vitamin intake is widely available in the literature (see Further Reading).

Sample Preparation

Vitamin A, the carotenoids, and vitamins E, D and K belong to the group of fat-soluble vitamins, which are soluble in organic solvents. The water-soluble vitamins B_1, B_2, B_6, B_{12}, C, biotin, folic acid, pantothenic acid, niacin, choline and inositol are soluble in water (**Table 1**). The structures of some fat-soluble and water-soluble vitamins are shown in **Figures 1** and **2.**

Sample preparation prior to the final chromatographic analysis is highly dependent upon the nature of the matrix. Minimal preparation is necessary for the analysis of concentrated solutions. For complex biological matrices more elaborate sample preparation procedures may be necessary. A 'recovery test' is highly recommended. This consists of adding a known amount of pure vitamin, approximatively equal to the estimated value in the sample, and processing the fortified sample in the same way as the sample itself. Loss of vitamin during analysis should not exceed 6%.

Fat-Soluble Vitamins

For fat-soluble vitamin assays all manipulations must be carried out in subdued light, in dark glass vessels, and in a nitrogen atmosphere to avoid isomerization and oxidation.

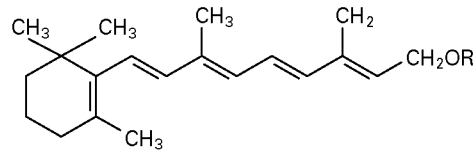
In foodstuffs, major interferences in assays for vitamin A, carotenoids, and vitamins E, D and K are caused by the large excess of other lipids. The vitamin A, carotenoids, and vitamins E and D contents are measured generally after alkaline hydrolysis with ethanolic KOH under a nitrogen stream at 60–80°C for 20–30 min in the presence of an antioxidant. Pyrogallol, hydroquinone, ascorbic acid (vitamin C) and butylated hydroxytoluene (BHT) are the most common antioxidants used during this manipulation. After saponification the free retinol, carotenoids, vitamin E and vitamin D are extracted into *n*-hexane or petroleum ether and evaporated to dryness. Vitamin A, carotenoids and vitamin E are redissolved in an organic solvent compatible with the chromatographic method to be employed. Vitamin K needs milder conditions for extraction from protein. Both vitamins D and K may require further purification before chromatography.

In the analysis of serum, vitamin A or retinol is liberated from its binding protein by denaturation with acetonitrile, ethanol or methanol. An internal

Table 1 Different kinds of vitamins

Fat-soluble vitamins	*Water-soluble vitamins*
Vitamin A (retionol)	Vitamin B_1 (aneurin, thiamin)
Carotenoids	Vitamin B_2 (riboflavin)
Vitamin D (ergocalciferol, vitamin D_2; cholecalciferol, vitamin D_3)	Vitamin B_6 (pyridoxine, pyridoxal, pyridoxine, pyridoxol)
	Vitamin B_{12} (cyanocobalamin)
Vitamin E (α-, β-, γ-tocopherols plus tocotrienols)	Vitamin C (ascorbic acid dehydroascorbic acid)
Vitamin K (phylloquinone, vitamin K_1; menadione, vitamin K_3)	Biotin
	Folic acid (vitamin B_9)
	Pantothenic acid (vitamin B_5, panthenol)
	Niacin (nicotinamide, vitamin PP)
	Choline
	Inositol (*myo*-inositol)

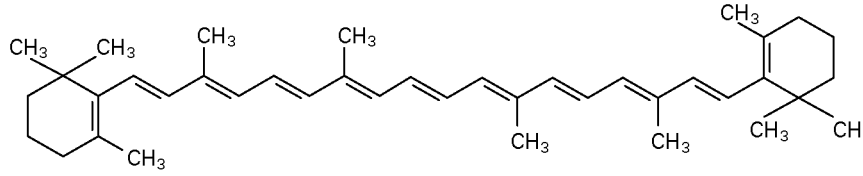
Vitamin A



Retinol	R = H
Retinylacetate	R = CO · CH_2
Retinylpalmitate	R = CO · $(CH_2)_{14}$ · CH_3

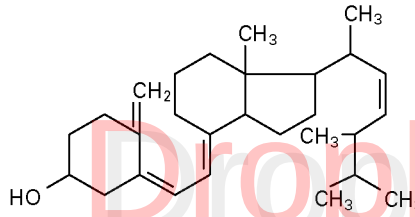
Provitamin A

β-Carotene

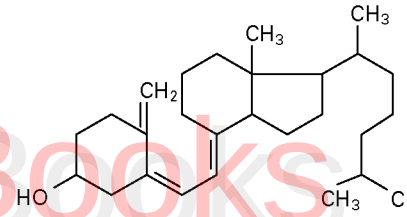


Vitamin D

Vitamin D_2
Ergocalciferol



Vitamin D_3
Cholecalciferol



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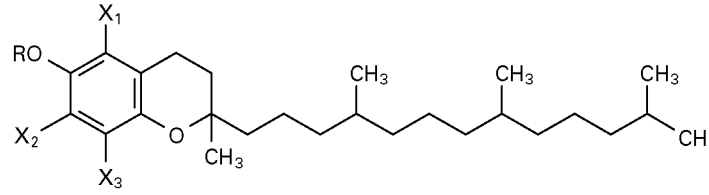
Vitamin E
Tocopherol



	X_1	X_2	X_3	R
α-Tocopherol	CH_3	CH_3	CH_3	H
β-Tocopherol	CH_3	H	CH_3	H
γ-Tocopherol	H	CH_3	CH_3	H
δ-Tocopherol	H	H	CH_3	H
α-Tocopherol acetate	CH_3	CH_3	CH_3	CO · CH_3

Vitamin K

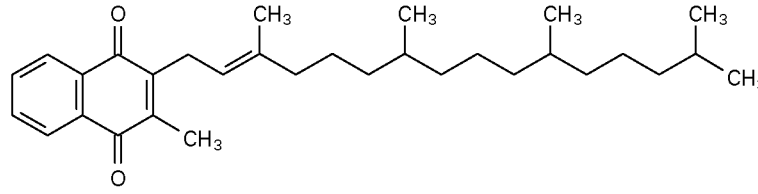


Figure 1 Chemical structures of some fat-soluble vitamins.

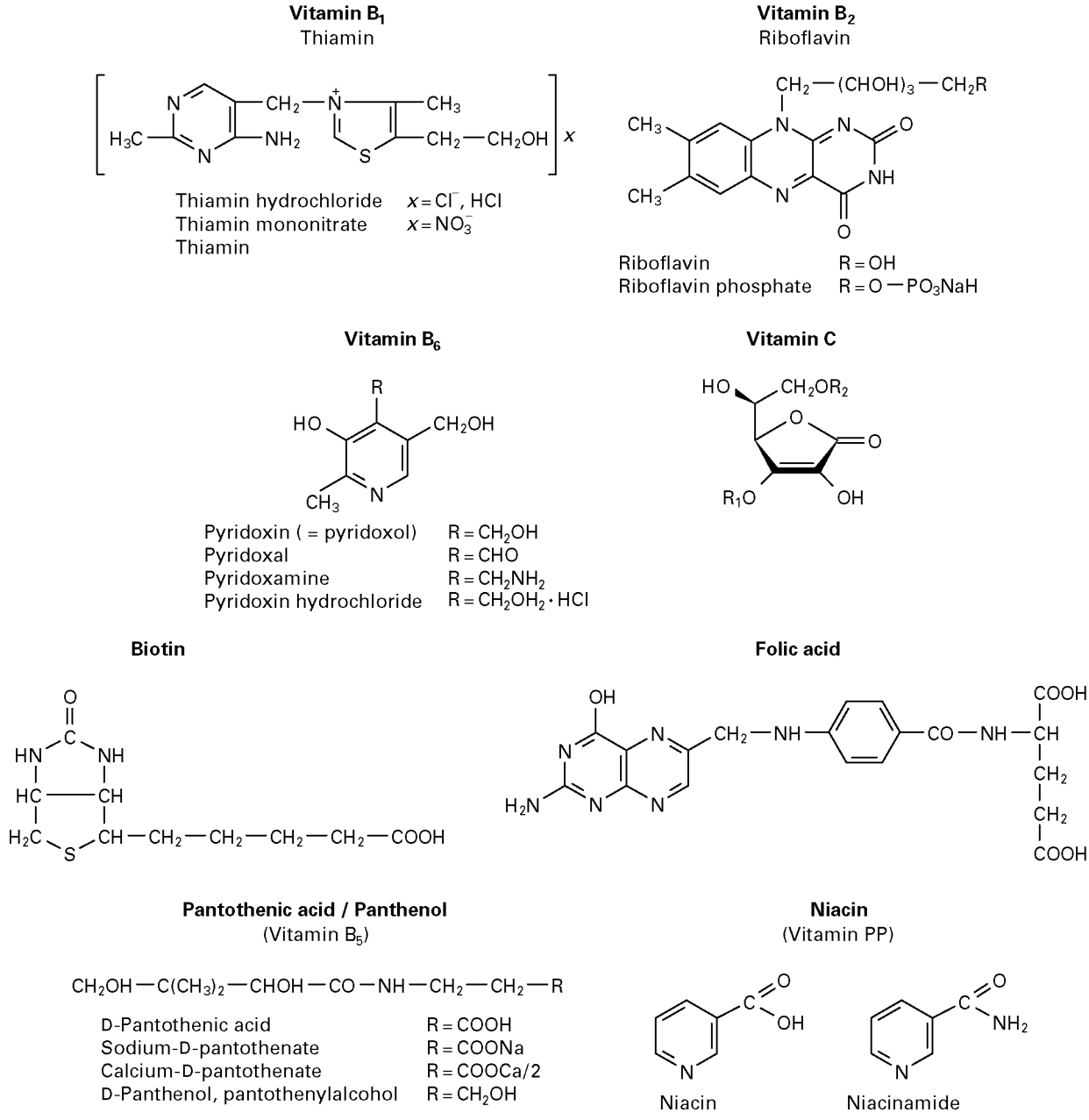


Figure 2 Chemical structures of some water-soluble vitamins.

standard, e.g. either retinyl acetate or tocol, is generally added for quantitation purposes. After protein precipitation the free retinol is extracted with 1% BHT in *n*-hexane solution, evaporated to dryness under nitrogen and subjected to chromatographic separation as above. Vitamin E and carotenoids are extracted in the same manner. Vitamins A, E and carotenoids can be simultaneously injected for LC separation. The analysis of vitamin D and trace quantities of vitamin D metabolites, e.g. 1,25-dihydroxycholecalciferol and 25-hydroxycholecalciferol, and also the analysis of vitamin K in human plasma, require an additional step for prepurification using column extraction or a semipreparative LC system prior to the final HPLC separation.

Water-Soluble Vitamins

Water-soluble vitamins are in general more stable than fat-soluble vitamins, although vitamin B_2 and to a lesser extent vitamin B_{12} (folic acid) are light sensitive. All manipulations should therefore be performed in subdued light. No special treatment of samples in

pharmaceutical preparations is required before chromatography. In biological fluids or foodstuffs prepurification and/or derivatization of the compounds are necessary before LC separation. The methods include acid extraction followed by enzymatic hydrolysis with takadiastase, papain or acid phosphatase, sometimes with pre-column or post-column chromatography. Trichloroacetic, perchloric and metaphosphoric acids are usually preferred for acid extraction. Depending on the aim of the investigation, vitamins can be determined in their free forms or in both free and phosphorylated forms. In the latter case the enzymatic hydrolysis step is omitted.

In blood, plasma and food, vitamin B_1 in protein-free extract is oxidized by agents such as $[Fe(CN)_6]^{3-}$, cyanogen bromide or mercuric chloride to thiochrome in a pre- or post-column chromatography reactor. For pre-column chromatography two different procedures are used. In the first, the thiochrome extract is neutralized by concentrated phosphoric acid to ensure a pH level compatible with the C_{18} column used for the separation and to eliminate possible pH-dependent alkaline degradation of thiochrome to its disulfide. It is then centrifuged and the supernatant injected into the HPLC. In the second procedure, isobutyl alcohol is used to extract thiochrome after alkaline oxidation. Aliquots of the extracts are then chromatographed.

After acid extraction vitamin B_2 is readily detected owing to its intense fluorescence.

Since vitamin B_6 is present in six chemical forms, there are methods for the simultaneous separation of the three free forms and the three phosphorylated forms as well as methods for determining the sum of all the forms. Pyridoxamine is transformed into pyridoxal by reaction with glyoxylic acid in the presence of Fe^{2+} as catalyst. The pyridoxal is then reduced to vitamin B_6 pyridoxol by the action of sodium borohydride in an alkaline medium before LC separation. Semicarbazide is also used for post-column derivatization of vitamin B_6.

In multivitamin–multimineral preparations, vitamin B_{12} or cyanocobalamin is extracted with a mixture of dimethyl sulfoxide (DMSO) and water, or ammonium pyrrolidine dithiocarbamate and citric acid in DMSO and water. The extract is centrifuged and the supernatant is diluted with water before concentration and clean-up by solid-phase extraction using a quaternary amine and a phenyl column in series before LC separation. There are few LC methods for the determination of vitamin B_{12} in human plasma and food.

In biological fluids and foodstuffs a treatment for removing protein is a major requirement for vitamin C assay. Protein precipitation may be done by organic reagents (methanol or acetonitrile) or mineral acids (perchloric, metaphosphoric acid, etc.). Aqueous solutions of vitamin C are rapidly oxidized on exposure to air. Stabilizers such as hydrogen sulfide and 1,4-dithio-DL-threitol have also been employed. The deproteinization may be followed by an enzymatic oxidation of ascorbic acid to dehydroascorbic acid, which is transformed with 1,2-phenylenediamine to its quinoxaline derivative for final separation.

Biotin, or vitamin H, is very stable. However, the limitation of HPLC lies in the lack of a suitable detection system. There are applications of LC to pharmaceutical products containing at least 300 μg biotin per tablet. In pharmaceutical products and feed premix, biotin is extracted from the matrix with buffer, followed by purification and concentration by solid-phase extraction and separation by LC. However, there are few LC methods for the estimation of biotin in biological samples.

Folic acid (pteroylglutamic acid; also called vitamin M) and its derivatives are stable substances. Folic acid may be determined simultaneously with other water-soluble vitamins in pharmaceutical preparations. In food products folates are extracted from the matrix with buffer and enzymes (e.g. hog kidney and chicken pancreas, or rat plasma conjugase, α-amylase and protease together), followed by purification and concentration by solid-phase extraction or with affinity chromatography before final separation.

In pharmaceutical preparations panthenol, panthotenic and its salt (vitamin B_5) are extracted with a phosphate solution. The extract is centrifuged, filtered and separated by LC. There are few methods for the determination of pantothenic acid and its salt in food products and biological fluids.

Niacin (or nicotinic acid) and nicotinamide are the two different forms of vitamin PP (so called for its pellagra-preventive factor). Nicotinamide is the form of the vitamin generally found in human plasma. Plasma is deproteinated with acetone/chloroform, the organic layer evaporated to dryness, and the methanolic extract of the residue separated by a reversed-phase HPLC. Isonicotinic acid is used as an internal standard. Urine is purified by extraction with chloroform, the aqueous phase evaporated, and taken for separation by LC. In foods vitamin PP is present mostly in its phosphorylated forms. Hydrolysis is necessary to break the ester bonds, releasing the total vitamin PP content of the food for assay. In food products niacin is extracted with buffer and enzyme. The sample extracts are purified through an ion exchange column (e.g. Dowex 1-X8 resin) before HPLC.

In multivitamin preparations 0.1 mol L^{-1} hydrochloric acid is used to extract the vitamins and

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DMSO containing anhydrous citric acid is used to disperse the multivitamin–multimineral preparation, since vitamin B_6 is not completely extracted by either 0.1 mol L^{-1} hydrochloric acid or DMSO owing to adsorption of the vitamin to the minerals. The extraction of nicotinamide is not impaired by the addition of citric acid to DMSO.

Choline in plant material is extracted with isopropanol containing internal standards and *p*-nitrobenzylhydroxylamine hydrochloride for the formation of *p*-nitrobenzyl oximes. The extract is purified by solid-phase extraction (C_{18} and ion exchange), after which the choline fraction is benzoylated to yield UV-absorbing derivatives. In biological samples choline is extracted with formic acid in acetone containing an internal standard. After purification the sample is separated by LC.

For the analysis of inositol mono- and diphosphate isomers in foods the method involves extraction of samples with hydrochloric acid and separation of inositol phosphates by anion exchange chromatography.

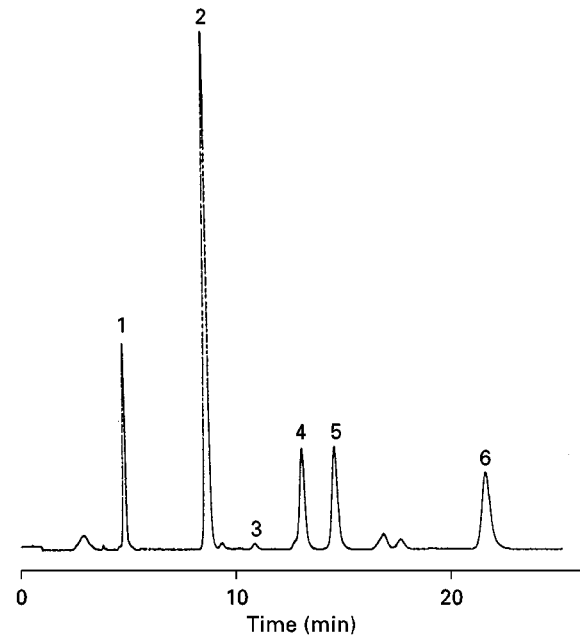

Figure 3 Example of an HPLC separation of α-, β-, γ- and δ-tocopherol from a wheat germ oil sample with α-tocopherol-acetate added. Peaks: 1, α-tocopherolacetate; 2, α-tocopherol; 3, α-tocotrienol; 4, β-tocopherol; 5, γ-tocopherol; 6, δ-tocopherol.

Experimental conditions: stationary phase, Lichrosorb Si 60, 7 μm; column dimension, 250 × 4.6 mm; mobile phase, *n*-hexane/dioxane (97 : 3 v/v); flow rate, 1.0 mL min^{-1}; injection volume, 20 μL; detection, fluorimetric with excitation at 295 nm and emission at 330 nm. (Reproduced with permission from Federal Office of Public Health, 1989.)

Liquid Chromatography

Liquid chromatography is an extremely valuable method for separation, identification and quantitation of the different vitamins. Excellent separations can be achieved in a reasonable time for routine analysis.

Fat-Soluble Vitamins

For fat-soluble vitamins normal-phase and reversed-phase chromatography are used.

In the normal-phase modes, silica and nonpolar mobile phases containing *n*-hexane or petroleum ether with a small percentage of a more polar solvent are used. Addition of a small amount of water or alcohol (e.g. ethanol) regulates the sorbent activity, reduces peak tailing and gives better reproducibility of retention times. Silica is the adsorbent of choice for the separation of *cis*/*trans* isomers and diastereoisomers. Selectivity on silica is determined by the number and the nature of the functional groups as well as the overall steric configuration (position of the double bonds) of the molecule (**Figure 3**).

In the reversed-phase mode, hydrophobic column packings (C_{18}, C_8, etc., bonded to a silica surface) are used together with an aqueous buffered mobile phase and a water-miscible organic solvent (i.e. methanol, acetonitrile).

Retinol analysis in biological fluids and foods is performed using both normal-phase and reversed-phase chromatography. For normal-phase (or liquid–solid) chromatography there is compatibility between the sample extraction solvent and the LC mobile phase; this avoids peak artefacts, especially for lipid extracts. Geometrical isomers such as 11-*cis*, 13-*cis*, 9-*cis* and all-*trans* retinol are well resolved. Retinol serum determination by reversed-phase chromatography allows the use of retinol acetate as internal standard which is well separated from retinol, unlike the case for liquid–solid chromatography. Meanwhile there is an additional step of evaporation of the extraction solvent in the sample preparation procedure before LC.

The most appropriate systems for the separation of polar and nonpolar carotenoids include the use of polymeric C_{18} or C_{30} bonded phases without end-capping in conjunction with a moderate pore-size packing column and a methanol-based mobile phase (to obtain a good recovery). *Cis*-isomers of β-carotene are largely resolved from each other and from other carotenes. Separation of lutein and zeaxanthine is also obtained with this system. Better separations of the xanthophylls are also observed. Accurate carotenoid measurements require the right selection of column and mobile phase and, due to the large degree of variability in the purity of commercial carotenoid

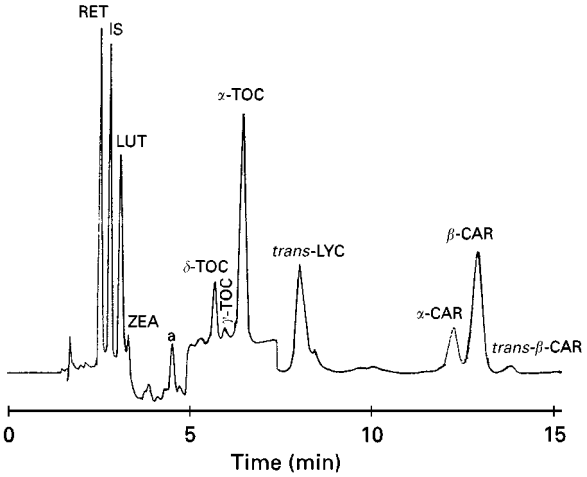

Figure 4 Chromatogram of a human serum sample. Peaks: RET, retinol; IS, retinol acetate (internal standard); LUT, lutein; ZEA, zeaxanthin; δ-TOC, δ-tocopherol; γ-TOC, γ-tocopherol; α-TOC, α-tocopherol; LYC, lycopene; α-CAR, α-carotene; β-CAR, β-carotene; *trans*-CAR, *trans*-β-carotene; *a*, unidentified carotenoid.

Experimental conditions: stationary phase. Lichrosorb RP-18, 7 μm; column dimension, 250 × 4.6 mm and 15 × 3.2 mm guard column; mobile phase, acetonitrile/tetrahydrofuran/methanol (68 : 22 : 7 v/v/v) adjusted to 100% with ammonium acetate; flow rate, 1.5 mL min^{-1}; injection volume, 15 μL; detection, programmable and variable-wavelength UV/Vis, 325 nm from 0 to 3.0 min, 450 nm from 3.0 to 4.9 min, 290 nm from 4.9 to 7.4 min, 470 nm from 7.4 to 12.0 min and 450 nm from 12.0 to 15.0 min. (Reproduced with permission from Bui, 1994.)

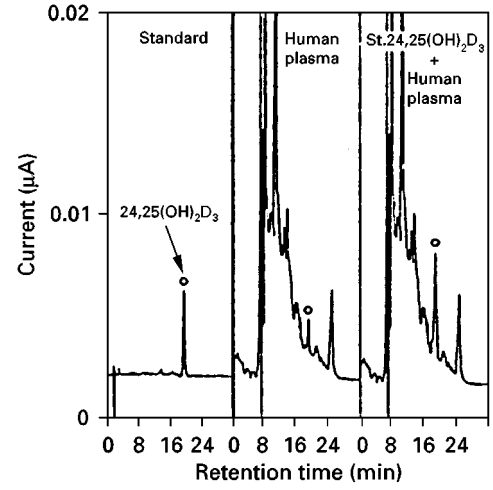

Figure 5 Chromatogram of HPLC-ECD for 24,25-dihydroxycholecalciferol (24,25-$(OH)_2D_3$).

Experimental conditions: stationary phase, Nucleosil C_{18}, 5 μm; column dimension, 300 × 7.5 mm; mobile phase, 5% (v/v) methanol in acetonitrile with 0.025 mol L^{-1} $HClO_4$; flow rate, 1.2 mL min^{-1}; injection volume, 50 μL; detection, dual-electrode electrochemical detection: detector 1 (+0.20 V); detector 2 (+0.60 V). (Reproduced with permission from Masuda *et al.*, 1997.)

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preparations, special precautions must be taken during calibration. Simultaneous determination of carotenoids, retinoids and tocopherols in serum and foods is performed on a C_{18} column using wavelength-programmable UV-visible (**Figure 4**), fluorescence or electrochemical detection.

The same separation conditions are used for the separation of α-tocopherol and retinol in biological fluids and foods. α-, β-, γ- and δ-Tocopherol as well as α-, β-, γ- and δ-tocotrienol are well resolved only by normal-phase chromatography. On an RP-18 column α-, β- and δ-tocopherol isomers are all separated, but not β- and γ-tocopherols. Tocol as internal standard is also used for vitamin E determination in serum.

Retinol and α-tocopherol in biological fluids, foods and pharmaceutical preparations can be separated simultaneously by normal- or reversed-phase HPLC.

In biological fluids analyses of vitamin D and its metabolites are performed using solid-phase extraction (SPE) cartridge coupled to semipreparative HPLC on a silica column and analytical HPLC on an octadecyl (C_{18}) column with UV or electrochemical detection (**Figure 5**). They can also be purified by one or two preparative HPLC steps on silica and quantified by HPLC on a C_{18} column with UV detection. Vitamin D_2 or D_3 may be used as internal standard (**Figure 6**).

The analysis of vitamin K (phylloquinone, menaquinone and epoxides), like vitamin D, uses normal-phase semipreparative LC followed by an analytical reversed-phase column with UV or electrochemical detection (**Figure 7**). Water-soluble menadione sodium bisulfite or vitamin K_3 in animal feeds is determined by reversed-phase HPLC with UV detection. To improve vitamin K_3 detection limits, post-column reaction fluorimetric detection is used. Menadione is hydrogenated by sodium borohydride to 2-methyl-1,4-dihydroxynaphthalene, which is detected fluorimetrically.

Water-Soluble Vitamins

Water-soluble vitamins are separated using ion exchange (IEC), normal-phase or reversed-phase chromatography.

Ion exchange chromatography is the preferred method of separation for the analysis of strongly ionic compounds. The chromatographic separation may be optimized by altering the pH or ionic strength of the mobile phase. Reversed-phase chromatography is the method of choice for water-soluble vitamins. Reversed-phase columns such as C_{18} and mobile phase NH_2 have been employed. The mobile phase is a mixture of methanol or acetonitrile with an acetate or phosphate buffer. For ionic compounds the reversed-phase ion pair mode is generally used. Unlike conventional

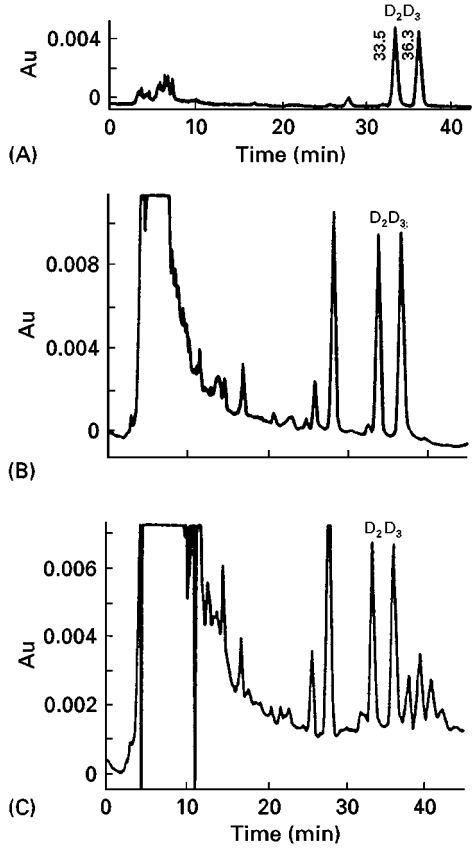

Figure 6 Analytical HPLC chromatograms of (A) standard mixture of ergocalciferol (D_2) and cholecalciferol (D_3); (B) D_2 (internal standard, IS) and D_3 in chicken sample; and (C) D_2 (IS) and D_3 in pork liver.

Experimental conditions: stationary phase, Zorbax ODS + Vydac 201 TP 54 5 μm; column dimension, 250 × 4.6 mm; mobile phase, 4% water in methanol; flow rate, 1.0 mL min^{-1}; injection volume, 50 μL; detection, UV at 264 nm. (Reprinted from Horvath CsG (ed.) (1980) *High Performance Liquid Chromatography*, New York: Academic Press. Copyright © 1980 by Academic Press.)

IEC, this technique can separate nonionic and ionic compounds simultaneously. The chromatographic separation may be optimized by altering the ion pair reagent, pH and ionic strength of the mobile phase.

Water-soluble vitamins (vitamin B_1, thiamin, B_2, riboflavin or riboflavin 5-monophosphate; B_6, pyridoxine and nicotinamide) in commercial vitamin preparations can be separated using either strong cation exchange resins or reversed-phase chromatography using an ion pair reagent (e.g. sodium alkane - sulfonate, dioctyl sodium sulfosuccinate, tetrabutyl ammonium phosphate) in the eluent with UV detection.

Similar LC methods are used for the separation of thiamin in foods and biological fluids, usually with fluorescence detection. In pre-column procedures, silica, C_{18}, NH_2 and poly(styrene–divinyl benzene) phases are used. Since the intensity of thiochrome fluorescence depends on pH and reaches a steady

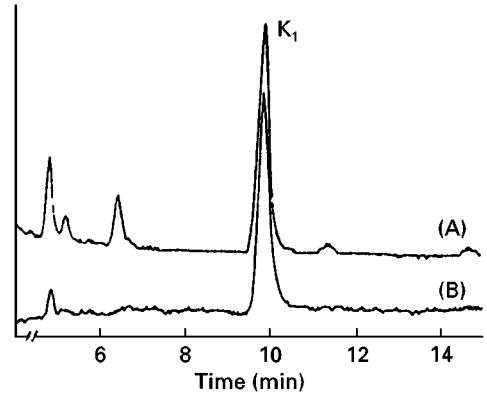

Figure 7 Analytical HPLC chromatogram of a kiwi fruit (A) without purification and (B) with purification with semipreparative HPLC.

Experimental conditions: stationary phase, Vydac 201 TP 548 5 μm; column dimension, 250 × 4.6 mm; mobile phase, 96% methanol/0.05 mol of sodium acetate buffer (pH 3); flow rate, 1.5 mL min^{-1}; injection volume, 30μL; detection, dual-electrode electrochemical detection: upstream electrode (−1.1 V); downstream electrode (0 V). (Reproduced with permission from Koivu *et al.*, 1997.)

level at pH > 8, the mobile phase should contain a buffer. Polymeric C_{18} packings are more suitable for these high pH conditions (**Figure** 8).

C_{18} columns are used for the determination of riboflavin and its derivatives, flavin mononucleotide (FMN) and flavin–adenine dinucleotide (FAD). The compounds are separated isocratically with a mixture

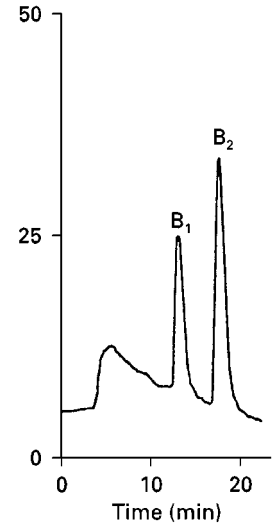

Figure 8 Chromatogram of thiamin (thiochrome) and riboflavin in a skimmed milk sample using fluorescence detection.

Experimental conditions stationary phase, Ultrasphere ODS 5 μm; column dimension, 250 × 4.6 mm; mobile phase, methanol/water (20 + 80) containing 0.005 mol L^{-1} tetrabutylammonium phosphate pH 7.5; detection, fluorimetric with excitation at 360 nm and emission 425 nm. (Reproduced with permission from Augustin, 1984.)

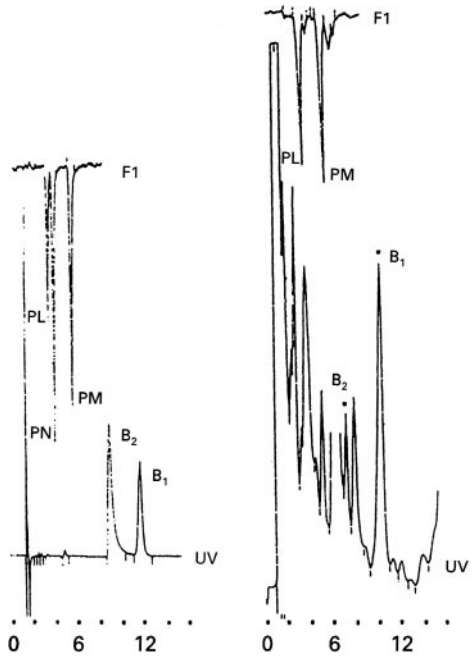

Figure 9 Chromatograms of B_1, B_2 and B_6 vitamers in standard and in whole blood.

Experimental conditions: stationary phase, Nova Pack C_{18} 5 μm; column dimension, 125 × 4.6 mm; mobile phase, 20% methanol in ion pair solution (at least 70 μL of di-*N*-butylamine solution per litre of the eluent); flow rate, 1.0 mL min^{-1}, detection, UV at 254 nm for B_1; fluorimetric with excitation at 290 nm and emission at 395 nm for B_2. (Reprinted from Setrell WH Jr and Harris RS (eds) (1967) *The Vitamins*, 2nd edn. New York/London: Academic Press. Copyright © 1967 by Academic Press.)

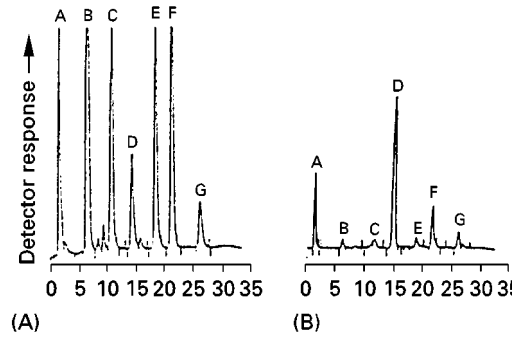

Figure 10 (A) Representative chromatogram of standard vitamin B_6 vitamer, 4-deoxypyridoxine (dPN) and 4-pyridoxic acid (4-PA). Peaks: A, pyridoxal phosphate PLP (8 pmol); B, 4-PA (10 pmol); C, pyridoxamine phosphate PMP (5.5 pmol); D, pyridoxal PL (10 pmol); E, pyridoxine PN (10 pmol); F, dPN (12 pmol); G, pyridoxamine PM (6 pmol). (B) Vitamin B_6 vitamer profile of human plasma.

Experimental conditions: stationary phase, Ultramex C_{18} guard column (30 × 4.6 mm) 3 μm and Ultramex C_{18} column (150 × 4.6 mm) 3 μm; mobile phase, (A) 0.033 mol L^{-1} phosphoric acid containing 0.01 mol L^{-1} 1-octanesulfonic acid adjusted to pH 2.2 with 6 mol L^{-1} potassium hydroxide; (B) 0.33 mol L^{-1} phosphoric acid in 10% (v/v) 2-propanol adjusted to pH 2.2 with 6 mol L^{-1} potassium hydroxide; flow rate, 1.2 mL min^{-1}; injection volume, 25 μL; detection, fluorimetric with excitation at 328 nm and emission at 393 nm. (Reprinted from Setrell WH Jr and Harris RS (eds) (1968) *The Vitamins*, 2nd edn. New York/London: Academic Press. Copyright © 1967 by Academic Press.)

of methanol and water (or buffer solution) using fluorimetric detection (**Figure 9**).

Simultaneous determination of the six chemical forms of vitamin B_6 in foods and biological samples is performed by IEC or reversed-phase chromatography,

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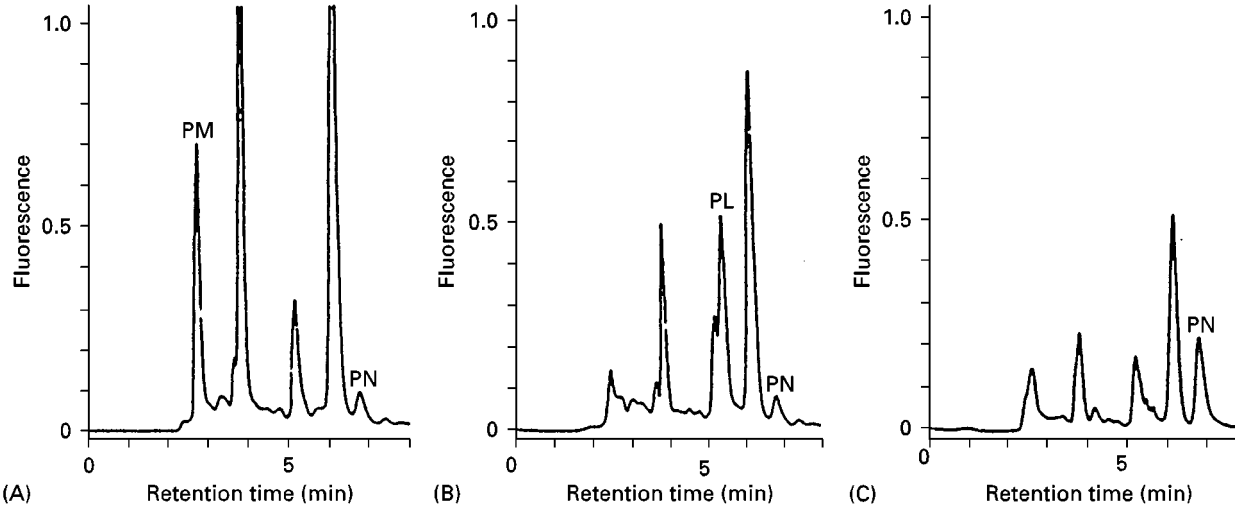

Figure 11 Chromatographic separation of vitamin B_6 vitamers in yeast. (A) With deletion of the deamination and reduction steps; (B) with deletion of the reduction step: (C) without deletion of either step.

Experimental conditions: stationary phase, Lichrospher 60 RP Select B octylsilyl 5 μm; column dimension, 250 × 5 mm; mobile phase, acetonitrile/0.05 mol L^{-1} potassium dihydrogen phosphate (4 : 96) containing 0.5×10^{-3} mol L^{-1} sodium heptanesulfonate adjusted to pH 2.5 with phosphoric acid; flow rate, 1.0 mL min^{-1}; injection volume, 20 μL; detection, fluorimetric with excitation at 290 nm and emission at 395 nm. (Reprinted from Setrell WH Jr and Harris RS (eds) (1972) *The Vitamins*, 2nd edn. New York/London: Academic Press. Copyright © 1972 by Academic Press.)

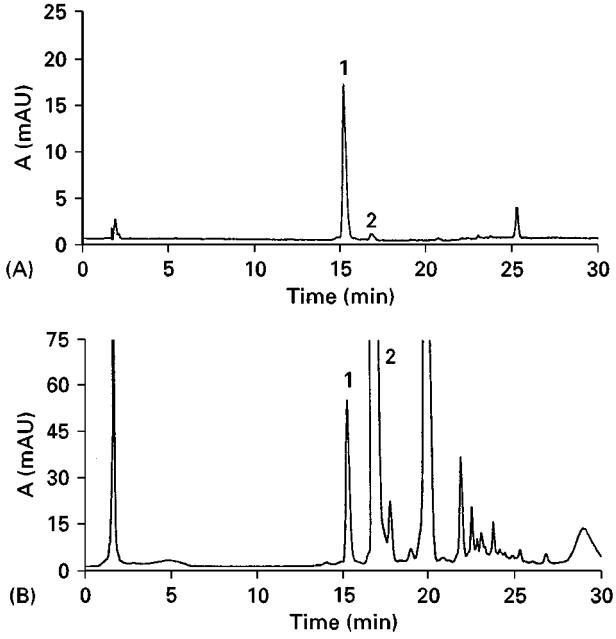

Figure 12 Chromatogram of multivitamin–multimineral tablets at (A) 50 nm and (B) 360 nm after preconcentration and clean-up by solid-phase extraction. Peaks: 1, vitamin B_{12}; 2, vitamin B_2.

Experimental conditions: stationary phase, Bondapack C_{18} 10 μm; column dimension, 150 × 39 mm; mobile phase, (A) methanol/water (10 : 90); (B) methanol/water (90 : 10); gradient elution, linear gradient to 50% B for the first 15 min, followed by 100% B for the next 2 min and maintained isocratically for 10 min; flow rate, 1.0 mL min^{-1}; injection volume, 200 μL; detection, UV at 550 nm. (Reproduced with permission from Dalbacke and Dahlquist, 1991.)

with or without ion pair reagents; detection is by fluorimetry (**Figure 10**). The vitamin B_6 content of foods can also be determined by ion pair HPLC after pre-column derivatization of the free and phosphorylated vitamin into pyridoxol (**Figure 11**).

Vitamin B_{12} is separated from other water-soluble vitamins in pharmaceutical preparations by reversed-phase using a methanol/water gradient with detection at 550 nm (**Figure 12**).

Reversed-phase chromatography is mostly used for ascorbic acid determination. In foods total vitamin C (ascorbic acid and its oxidized form, dehydroascorbic acid) are determined using ion pair chromatography with UV detection. In biological fluids and foods total vitamin C, as its quinoxaline derivative, is separated on a C_{18} column with fluorescence detection. The determination of ascorbic acid in plasma can also be achieved using a C_{18} column and electrochemical detection. Another procedure for vitamin C determination consists of first measuring the ascorbic acid present, then reducing the dehydroascorbic acid, at neutral pH, with dithiothreitol, and finally measuring the total ascorbic acid. The dehydroascorbic acid is determined by difference. The separation is on a C_{18} column with electrochemical detection.

After a clean-up procedure, biotin in pharmaceutical products is assayed using a C_{18} column with methanol/water as the mobile phase and UV detection. Extracts of folates (folate monoglutamates and folic acid) in food and biological samples after purification are separated by gradient elution and UV or fluorescence detection (**Figure 13**).

Pantothenic acid is separated from other water-soluble vitamins with an isocratic system on an aminopropyl bonded phase using a mixture of acetonitrile/phosphate buffer as mobile phase and UV detection (**Figure 14**). Panthenol in multivitamin

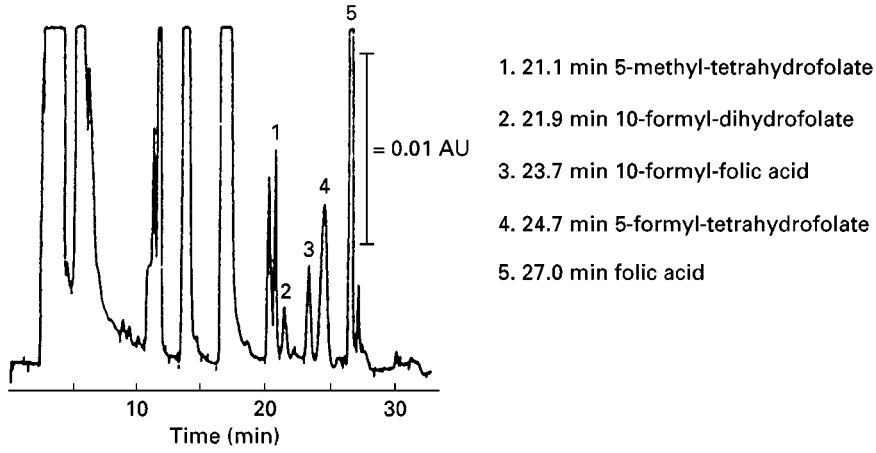

Figure 13 Chromatogram of the main folate forms found in fortified white bread.

Experimental conditions: stationary phase, Phenomenex Ultramex C_{18} 5 μm; column dimension, 250 × 4.6 mm; mobile phase, 33 mmol L^{-1} phosphoric acid, pH 2.3 with increasing acetonitrile; gradient elution, 5% (v/v) acetonitrile maintained isocratically for the first 8 min, linear gradient to 17.5% (v/v) within 25 min; flow rate, 1.0 mL min^{-1}; detection, UV at 280 nm. (Reproduced with permission from Pfeiffer *et al.*, 1997.)

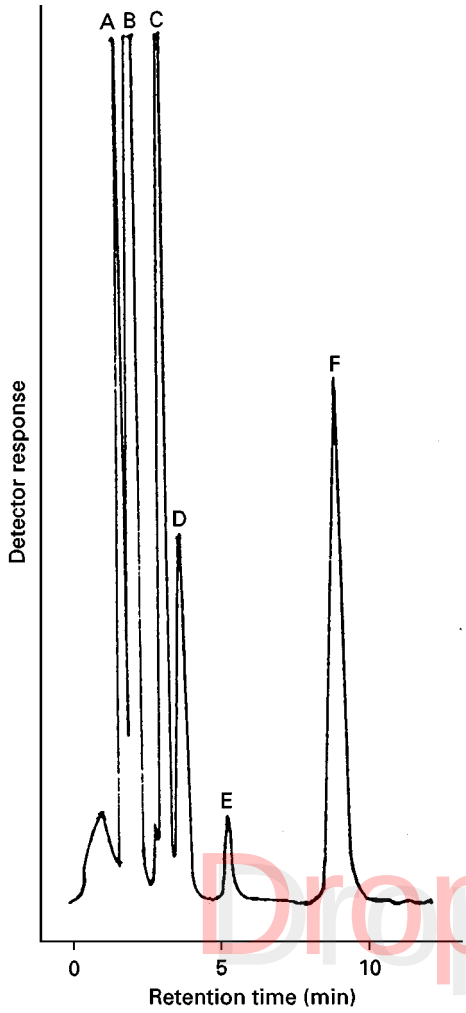

Figure 14 Chromatogram of a high potency B complex tablet extract. Peaks: A, niacinamide; B, vitamin B_6; C, vitamin B_2; D, vitamin B_{12}; E, unknown; F, pantothenic acid.

Experimental conditions: stationary phase, Hibar II Lichrosorb NH_2 10 μm; column dimension, 250 × 4.6 mm; mobile phase, 0.005 mol L^{-1} monobasic potassium phosphate (pH 4.5)/acetonitrile (13 : 87 v/v) 0.01 mol L^{-1} 1-octanesulfonic acid; flow rate, 2.0 mL min^{-1}; injection volume, 10 μL; detection, UV at 210 nm. (Reproduced with permission from Hudson and Allen, 1984.)

preparations is determined on a C_{18} column with a gradient system using an ion pair reagent (e.g. sodium hexanesulfonate) and UV detection. Niacin and nicotinamide are also separated on a C_{18} column using an ion pair reagent with UV detection. Isonicotinic acid is used as an internal standard.

Cation exchange chromatography has been used to determine with UV or electrochemical detection. Separations of inositol mono- and diphosphate isomers in foods is performed on an anion exchange column using a sodium acetate in sodium hydroxide gradient with electrochemical detection.

Future Developments

Liquid chromatography is the method of choice for vitamin analysis in pharmaceutical products, foods, feeds and especially in biological fluids. In biological sample analysis LC affords separation of the vitamins, their related compounds and various metabolites for nutrition research. The main problem encountered in biological materials is the detection limit, particularly for water-soluble vitamins. There are two main areas where developments are necessary for future vitamin LC. First, automatic sample preparation techniques involving vitamin purification and enrichment, e.g. automating solid-phase extraction, need to be improved. Second, the coupling LC and mass spectrometric (MS) detection needs to be further developed. These techniques may become leading methods in vitamin analysis in the future.

See also: **II/Chromatography: Liquid:** Mechanisms: Normal Phase; Mechanisms: Reversed Phases. **III/Carotenoid Pigments: Supercritical Fluid Chromatography. Food Technology:** Supercritical Fluid Chromatography. **Vitamins:** Fat-Soluble: Thin-Layer (Planar) Chromatography; Water-Soluble: Thin-Layer (Planar) Chromatography.

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Water-Soluble: Thin-Layer (Planar) Chromatography

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As a tool, chromatography has long been important for the separation of vitamins from complex mixtures and their initial isolation and identification would have been greatly hampered without the use of paper, column or thin-layer chromatography (TLC). While more sophisticated chromatographic techniques are now widely available, TLC has great advantages in terms of its simplicity and flexibility of use.

The vitamins classified as water-soluble are all compounds important in human metabolism either as coenzymes or their precursors which the body cannot make for itself (**Figure 1**). The recommended daily allowance (RDA) of each vitamin ranges from hundreds of milligrams to just a few micrograms a day (**Table 1**). These compounds have few properties in common apart from their water-solubility, but this fact alone makes TLC an excellent technique for their separation, particularly in pharmaceutical preparations and food products. Even at physiological concentrations, TLC is widely used after extraction of the vitamins from tissues or body fluids. This generally needs to be under acid conditions. Since most of these compounds are unstable at high pH. Some are in addition very light-sensitive. Following TLC separation, special methods of detection may also be required, since tissue levels of most water-soluble vitamins are low or very low.

Thiamin (Vitamin B_1)

Thiamin occurs in plant and animal tissues and the richest sources are seeds and nuts, peas and beans, cereals and yeast. Fish and meat, notably pork, are also good sources. Thiamin is commonly available as its monohydrochloride, but it also forms acid salts and esters with nitric and phosphoric acids. Metabolically, thiamin is required as the coenzyme thiamin pyrophosphate for the mitochondrial metabolism of glucose and pyruvate.

Thiamin may be extracted from tissues, foodstuffs or pharmaceutical preparations with aqueous alcohol mixtures at a pH of 4–6 and separated from closely related compounds and metabolites by TLC on cellulose or silica gel. Various mobile phases have been successfully used, including isopropanol–water–trichloracetic acid–ammonia (71 : 9 : 20 : 0.3) and butan-1-ol–acetic acid–water (40 : 10 : 50). Thiamin may be separated from its hydrolysis and oxidation products by TLC/densitometry and other chromatographic techniques have been reviewed. Sandwich-type chambers afford rapid separation of thiamin from other water-soluble vitamins by TLC on silica gel GF254 and the spots then located under UV light. An alternative technique for the quantitation of thiamin in pharmaceutical products involves the use of high performance TLC (HPTLC) and post-separation derivatization with a hexacyanoferrate (III)-sodium hydroxide reagent and fluorodensitometry, sensitive down to 500 pg per spot. Other modifications include the use of a fibreoptic probe